Validation of serine/threonine protein phosphatase as the herbicide target site of endothall

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A B S T R A C T

Endothall, an older commercial herbicide, and cantharidin, a natural product from the blister beetle (Epicauta spp.), are close chemical analogues. A comparison of the effect of endothall and cantharidin on plants revealed a similarity in their level of phytotoxicity on both Arabidopsis thaliana and Lemna paucicostata. Cantharidin is a potent inhibitor of animal serine/threonine protein phosphatases. Protein phosphatases and kinases maintain a sensitive balance between phosphorylated and dephosphorylated forms of proteins playing important roles in signal transduction pathways. In this study, we investigated endothall and cantharidin to both completely inhibit plant serine/threonine protein phosphatases, and their relative inhibitory activities were similar to their relative phytotoxicities. Both compounds acted as slow, irreversible inactivators of the serine/threonine protein phosphatase activities. Transcription of several genes determined to be affected by the inhibition of these protein phosphatases by cantharidin in A. thaliana by transcriptome analyses were affected similarly by endothall, but in a more pronounced way. Therefore, the molecular target site of endothall in plants is similar to that of cantharidin in animals, namely, serine/threonine protein phosphatases responsible for regulating an array of biochemical processes. This mode of action is unlike any other commercial herbicide.

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1. Introduction

Endothall ([7-oxabicyclo[2.2.1]heptane-2,3-dicarboxylic acid, Fig. 1] was introduced in the 1950s, yet it is one of the few herbicides still listed as having an unknown mode of action [1,2]. In a chemical class of its own, endothall induces symptoms similar to chilling injury on the foliage of treated plants and ultimately results in severe growth inhibition [2]. Investigations on the mode of action of endothall reported effects on mRNA [3] and protein [4] synthesis. Additionally, endothall causes membrane dysfunction [5], and the WSSA Herbicide Handbook suggests that its action may initiate at the membrane level [2]. However, loss of membrane integrity can occur in plants exposed to herbicides with modes of action involving a wide array of molecular target sites [6,7]. The primary target site of endothall in plants has remained elusive for many years.

Endothall is a close analog of cantharidin (Fig. 1) from the blister beetle (Epicauta spp.) and the Spanish fly (Lytta vesicatoria). Some insects accumulate up to 5% of their dry weight of this natural defense terpenoid. Cantharidin has had various uses as a pharmaceutical over the years (e.g., [8–10]), and is infamous as an illicit aphrodisiac associated with human poisonings [11,12]. It is currently being investigated as an anticancer drug lead [13]. In animals, cantharidin is a strong inhibitor of serine/threonine protein phosphatases (PPs) [14], a broad class of PPs associated with signaling and control of numerous cellular processes in many organisms (e.g., [15]). The catalytic domain of all PP subfamilies is highly conserved in animals, plants and protozoans [16]. Inhibitors, such as cantharidin and endothall, bind to a hydrophobic pocket of the catalytic domain of animal Ser/Thr PPs or the plant PPs [14]. In plants, cantharidin is a strong inhibitor of serine/threonine protein phosphatase 2A (PP2A) activity, and endothall is a potent inhibitor of plant serine/threonine protein phosphatases [17]. endothall and cantharidin have similar modes of action on animal and plant serine/threonine PPs. The structural similarity between endothall and cantharidin has been apparent to animal scientists and similar mechanisms of action on animal serine/threonine PPs have been confirmed (e.g., [18]).

Scientists often use herbicides as molecular probes to study plant biochemical processes [19]. Endothall has recently been used to manipulate serine/threonine PP activity and investigate a number of plant processes including disease resistance [20–24], metal ion toxicity [25,26], apoptosis [27], and regulation of anthocyanin biosynthesis [28]. Endothall strongly inhibits alfalfa serine/threonine PP2A (PP2A) activity, as well as serine/threonine PPI (PP1) to a lesser degree, which is accompanied by a profound effect on...
on plant cell coordination of chromosomal and microtubule events during mitosis [29]. Tresch et al. [30] recently connected the inhibition of serine/threonine PP2As by endothall to its pronounced effects on the plant cell cycle, which were similar to phenotypic effects on plants with a mutant regulatory subunit (TOS1) of a protein phosphatase (PP2A) in Arabidopsis thaliana. However, the phytotoxicity of endothall was not directly compared to its effect on serine/threonine PP activity. We previously reported that cantharidin was highly phytotoxic, apparently due to its effects on plant serine/threonine PPs [31,32]. Herein, we validate the relationship between the herbicidal activity of endothall and inhibition of serine/threonine PPs supporting this data with an analysis of the compound's effects on the expression of selected A. thaliana gene transcripts.

2. Materials and methods

2.1. Chemicals

Technical grade endothall, cantharidin and all other chemicals for the experiments were obtained from Sigma–Aldrich (St. Louis, MO, USA), with the exception of Tween 20 (Fisher Scientific, Pittsburgh, PA, USA) and Murashige and Skoog (MS) basal salt mixture (Phytotechnologies Laboratories, Shawnee Mission, KS, USA).

2.2. Culture and treatment of plants

Wild-type A. thaliana Columbia ecotype was grown on MS medium, five seedlings per well of a six-well plate at 24 °C and under constant light intensity of 100 µmol m⁻² s⁻¹ PAR. Twelve-day-old seedlings were sprayed with a solution of 0.1% acetone and 0.15% Tween 20 per container. Controls were sprayed with a solution of 0.1% acetone and 0.15% Tween 20 solution. The spray solution was applied to cover the leaf tissue entirely. Two days after spraying, shoots of the seedlings were harvested and chlorophyll content was determined by the method of Brouwer [43].

3. Results

3.1. Treatment of plants

Three independent replicates of both treated and untreated (control) samples were conducted as previously described [35]. L. paucicostata stocks were grown on modified Hoagland media. The media contained: 1515 mg L⁻¹ KNO₃, 680 mg L⁻¹ KH₂PO₄, 492 mg L⁻¹ MgSO₄·7H₂O, 20 mg L⁻¹ Na₂CO₃, 1180 mg L⁻¹ Ca(NO₃)₂·4H₂O, 0.5 mg L⁻¹ H₂BO₃, 0.05 mg L⁻¹ ZnSO₄·0.12 mg L⁻¹ Na₂MoO₄·0.47 mg L⁻¹ MnCl₂·0.025 mg L⁻¹ CoCl₂·0.025 mg L⁻¹ CuSO₄·18.35 mg L⁻¹ Fe-EDTA. The medium was adjusted to pH 5.5 with 1 M NaOH and then filter sterilized through a 0.2 µm sterile filter. All of the L. paucicostata used in these studies originated from a single L. paucicostata colony (an aggregate of one mother and two daughter fronds) to assure genetic uniformity. For the tests, plants were taken while still in exponential growth from a 4 to 5-day-old stock culture.

Each concentration of a compound was tested in three replicates. If required, the pH of the media was adjusted to 5.6 after adding test chemicals. Both the initial screening and replicate series tests were conducted in an incubator (Model #CU-36L, Percival Scientific, Boone, IA, USA) with white light (94 µE m⁻² s⁻¹) using non-pyrogenic polystyrene sterile six-well plates (Corning Inc., Corning, NY, USA) with a lid. Each well contained 4950 µL of the Hoagland media plus 50 µL of water, or the solvent (as control), or the appropriate concentration of the compound dissolved in a suitable solvent. The final concentration of the solvent was therefore 1% by volume. Each well was inoculated with two, three-frond colonies of approximately the same size. The selected colonies had a “Mickey Mouse” appearance, where the developing daughter fronds were significantly smaller and still attached to the maternal frond. Total frond area per well was recorded by the image-analysis-system Scanalyser (LemnaTec, Würselen, Germany) once per day from day 0 to day 7.

2.3. Protein phosphatase assays

One gram of freeze-dried plant material was ground in a mortar and pestle and suspended in 25 mM Tris–HCl pH 7.0, 0.1% β-mercaptoethanol, 1 mM EDTA, 1 mM benzamidine, and 0.05% Tween 20 and plant protease inhibitors cocktail (Sigma–Aldrich). The tissue was homogenized with a Polytron instrument (PT 3100, Kinematica AG, Littau-Lucerne, Switzerland) and centrifuged at 17,500g for 30 min. The cell free extract was desalted over a Sephadex G25 spin column to remove contaminating free phosphate. Protein concentration was determined by the Bradford method [36]. Serine/threonine PP activity in the extracts was measured using a serine/threonine PP assay system kit (Promega, Madison, WI, USA) in half-area 96-well plates (Corning Inc.). Dose-response curves of the effect of cantharidin and endothall were performed on desalted, cell-free extracts isolated from A. thaliana or L. paucicostata seedlings. Reactions were incubated with predetermined concentrations of inhibitors at room temperature for 15 min, after which the serine/threonine peptide substrate was added producing an overall reaction volume of 50 µL. The reaction was carried out for 30 min, subsequently stopped by adding 50 µL of molybdate dye/additive mixture and incubated at room temperature for an additional 15 min for color development. The absorbance of a molybdate/malachite green phosphate complex was measured with a PowerWave XS plate reader (Bio-Tek, Winooski, VT, USA), with a 600 nm filter and software (KC Junior, version 1.41.a).
The mechanism of interaction between the inhibitors (endothall and cantharidin) with their molecular target sites was evaluated via two assays. First, a time-course study checked the serine/threonine PP activity in 

\[ L. paucicostata \] total soluble protein extracts upon pre-incubation with the inhibitors for 0, 5, 10, 20, 30, 40, and 50 min. The assay described above contained 2 \( \mu \)g total soluble protein per assay. A subsequent experiment was performed by titrating the amount of total soluble protein per assay from 1 to 6 \( \mu \)g per assay.

2.4. Gene transcript analysis

Total RNAs were isolated using the Trizol reagent (Invitrogen, Carlsbad, California, USA) according to manufacturer’s instructions. The RNA samples were treated with DNase I using an RNase-Free DNase kit as per the manufacturer’s instructions (Qiagen, Valencia, CA, USA) to remove residual DNA contamination. The RNA recovered was then re-purified with the RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). The integrity of purified total RNA was established by 1% agarose gel electrophoresis using ethidium bromide staining. The concentration and purity of total RNA was determined spectrophotometrically.

First strand cDNAs were synthesized from total RNA using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) as per the manufacturer’s instructions. Quantitative real time PCR was performed in triplicate using the IQ SYBR Green Supermix (Bio-Rad) for double-stranded DNA synthesis, and the fluorescence signal was monitored on line using a Bio-Rad MiniOpticon real-time PCR detection system (Bio-Rad) according to the manufacturer’s directions. Primers for PCR reaction were designed and analyzed using Primer Express Software (Table 1). Specificity and exclusivity of chosen primers was determined using BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) against the A. thaliana genome database. RNA levels were normalized using Actin 2 (At3g18780) as a reference gene. Efficiencies were calculated using a 10-fold serial dilution series of DNA. Reaction conditions were as follows: initial denaturation 95 °C for 2 min, followed by forty cycles of 95 °C for 20 s, 60 °C for 10 s, 72 °C for 30 s, and a subsequent standard dissociation protocol to check for the specificity of primers to the genes of interest. Results were analyzed using the Bio-Rad CFX Manager software (version 2.0). PCR products were also assessed by electrophoresis on 1.5% agarose gel.

2.5. Statistical analysis

Data from dose–response experiments were analyzed using the dose–response curve (DRC) module [37] and R version 2.2.1 [38]. Means and standard deviations were obtained using the raw data, and I_{so} values were obtained from the parameters in the regression curves. The regression curves were imported into SigmaPlot version 10 (Systat Software Inc., San Jose, CA, USA). Pearson correlation coefficients were calculated using the statistical module of SigmaPlot.

3. Results

3.1. Symptoms and relative phytotoxicity of endothall and cantharidin

A. thaliana seedlings developed visible symptoms, such as reduced growth, loss of chlorophyll, and hypersensitive response-like lesions within the first 24 h after treatment. The endothall and cantharidin concentrations that reduced chlorophyll in A. thaliana by 50% \( (I_{so}) \) were 1.36 ± 0.22 and 0.73 ± 0.08 mM, respectively, after two days of treatment (Fig. 2A). A. thaliana seedlings growing on media containing endothall or cantharidin were more sensitive to these compounds, which reduced root growth by 50% at 13.9 ± 0.73 and 73.2 ± 5.11 \( \mu \)M, respectively (Fig. 2B). Endothall and cantharidin were even more potent against the growth of L. paucicostata, with I_{so} values of 10.0 ± 1.9 and 0.24 ± 0.02 \( \mu \)M, respectively (Fig. 2C).

3.2. Effects on serine/threonine protein phosphatase activity

Endothall and cantharidin inhibited serine/threonine PP activity in a total soluble protein extract from A. thaliana seedlings. The apparent 50% inhibition \( (I_{so}) \) app of endothall and cantharidin in an in vitro serine/threonine PP assay were 2.0 ± 0.3 and 0.6 ± 0.07 \( \mu \)M, respectively (Fig. 3A). The dose/response curves

Table 1

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Locus</th>
<th>Gene description</th>
<th>Primer sequencea</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACT2</td>
<td>At3g18780</td>
<td>Actin 2</td>
<td>F: 5' - TGGCAACATCACCATTGCATGTC - 3'</td>
</tr>
<tr>
<td>WRY6</td>
<td>At1g02300</td>
<td>Transcription factor for phosphate1 (Pho1) expression</td>
<td>R: 5' - TACCTACACAACCAGTTGTCG - 3'</td>
</tr>
<tr>
<td>ASA1</td>
<td>At5g05730</td>
<td>Alpha subunit of anthranilate synthase</td>
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</tr>
<tr>
<td>DREB</td>
<td>At5g25810</td>
<td>DREB subfamily A-4 of ERF/AP2 transcription factor family (TINY)</td>
<td>R: 5' - AAGCCGGGACCGGCGTCGGATAC - 3'</td>
</tr>
<tr>
<td>ACS6</td>
<td>At4g11280</td>
<td>1-Aminoclopropane-1-carboxylate (ACC) synthase</td>
<td>F: 5' - CTCTTCTGCTTTCCTCCTGCTG - 3'</td>
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<tr>
<td>T14L22.10</td>
<td>At1g51890</td>
<td>Leucine-rich repeat protein kinase family protein</td>
<td>R: 5' - TCCGGCCTTGGGCGGTCGGGCTGCA - 3'</td>
</tr>
<tr>
<td>PKS1</td>
<td>At2g02950</td>
<td>Soluble protein binding to PHYA or PHYB</td>
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<tr>
<td>GAS1A</td>
<td>At1g75750</td>
<td>GA-responsive GAST1 protein homolog</td>
<td>R: 5' - CCGCGGATGCTGCTGCTGCTG - 3'</td>
</tr>
<tr>
<td>C3CH4</td>
<td>At5g27420</td>
<td>CN1 RING type ubiquitin ligase</td>
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<td>At1g29510</td>
<td>Auxin responsive SAUR protein</td>
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<tr>
<td>RPM1</td>
<td>At3g07040</td>
<td>Disease resistance protein RPM1</td>
<td>R: 5' - AGCTGCCCTGGGCGGCTAAGATG - 3'</td>
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* F indicates forward and R indicates reverse.
were very broad for both inhibitors, with complete inhibition of serine/threonine PP at about 100 µM. This may be due to the crude extract containing a number of different serine/threonine PPs with a range of sensitivities to the inhibitors. These compounds were more active on serine/threonine PP activity extracted from *L. paucicostata*, with $I_{50}$ values of 1.3 ± 0.05 and 0.068 ± 0.005 µM for endothall and cantharidin, respectively (Fig. 3B).

The inhibition exerted by these compounds on serine/threonine PP activity in *L. paucicostata* total soluble protein extracts increased over time (Fig. 4A). The onset of inhibition was more rapid with cantharidin than with endothall (when tested at their respective $I_{50}$ concentrations). Titration of the amount of protein against a constant concentration of either endothall or cantharidin in the reaction assay (Fig. 4B) supports the time-dependent inactivation of serine/threonine PP. However, the lines were not completely parallel.

### 3.3. Effects on gene transcription

Since the RT-PCR experiment required *A. thaliana* to be exposed to endothall and cantharidin for relatively short period of times, growing the seedlings on plates as was done for the root length experiment (Fig. 2B) was not an option. Therefore, seedlings were sprayed with a 200 µM of each compound, a concentration that was near the beginning of phytotoxicity on the dose-response curve for both compounds. After spraying, samples were taken at 2, 10 and 24 h.

The effects of endothall and cantharidin on *A. thaliana* gene transcription were compared by quantitative real-time RT-PCR
assays. Eleven genes whose expression responded to cantharidin in a previous microarray transcriptome analysis [31,32] were selected for this study (Table 1). Genes were chosen based on their association with significant functions, and, additionally, some of these were among those most affected by cantharidin. These functions included various types of defense responses and hormonal, and phytochrome signaling, such as hypersensitive response (RPM1 (AT3G07040)); flagellin signaling (WRKY6 (AT1G62300)); ethylene signaling (DREB (At5g25810), ACS6 (At4g11280) and ASA1 (AT5G05730)); auxin responses (PBP1 (At5g54490) and SAUR68 (At1g29510); and phytochrome signaling (PBP1 (AT1G51890) and PKS1 (AT2g02950)). RNAs extracted from cantharidin and endothall treated seedlings were analyzed. Statistical analysis revealed a strong correlation between the genes' responses to cantharidin and endothall treatments, which is consistent with the two compounds having similar effects on plants (Fig. 5). The Pearson correlation coefficient was highest after 2 h of exposure to the compounds (Fig. 5A). The strength of the correlation decreased after 10 h (Fig. 5B), but appeared to be restored after 24 h of exposure to the herbicides (Fig. 5C).

4. Discussion

Endothall and cantharidin were more active on L. paucicostata than A. thaliana (Fig. 2A and B), which is in agreement with a previous study showing that monocots are more sensitive than dicots [39]. The relative effects of cantharidin and endothall as
phytotoxins on *A. thaliana* and *L. paucicostata* (Fig. 2) were similar to their effects on serine/threonine PP enzyme activity (Fig. 3). Cantharidin was more active than endothall as a phytotoxin on chlorophyll content of *A. thaliana* and growth of *L. paucicostata* and as an enzyme inhibitor, especially in *L. paucicostata*. This implies a connection between inhibition of serine/threonine PP activity (up to 19 forms of the enzyme in *A. thaliana* are presumed to be inhibited by cantharidin [31]) and phytotoxicity.

Endothall and cantharidin inhibited serine/threonine PP activity in total soluble protein extracts from *A. thaliana* and *L. paucicostata*. In all instances, cantharidin was more potent than endothall, and inhibition was greater on *L. paucicostata*. This suggests that there may be differences between serine/threonine PPs from dicotyledonous and monocotyledonous species (Fig. 3A and B). However, little is known about the interactions of these compounds with serine/threonine PP from different plant species.

The anhydride-type serine/threonine PP inhibitors are more active than their corresponding di-acids in vitro, which has led to some debate about whether cantharidin or its hydrolyzed form, cantharidic acid (Fig. 1), is the serine/threonine PP inhibitory form of the compound. However, analogues capable of undergoing rapid ring opening of the anhydride moiety were more active than derivatives that could not undergo such a reaction [40], and a study finally demonstrated that the anhydride rings of both cantharidin and norcantharidin are hydrolyzed when bound to the catalytic domain of the human serine/threonine PP [17]. Cantharidic acid is a closer analog to the di-acid endothall than cantharidin in that it varies by only two additional methyl groups (Fig. 1). However, the presence of these substituents contribute significantly to the overall activity of these inhibitors [41,42]. This is consistent with our data showing that cantharidin is a more potent inhibitor of plant serine/threonine PP (Fig. 3).

The time-dependent interaction between the inhibitors and serine/threonine PP (Fig. 4A) suggests that they are slow inactivators of these enzymes. This is in agreement with a previous report showing a marked time-dependent inhibition of serine/threonine PP activity by okadaic acid [43]. Typically, enzyme activity is linearly correlated with enzyme concentration under conditions in which no other component of the reaction is limiting, as can be observed with the control plot in Fig. 4B. In such enzyme titration assays, reversible inhibitors cause the lines to resolve toward the origin, whereas compounds that bind irreversibly to enzymes will generate parallel lines, as can be observed with some HPPD inhibitors [44,45]. In our case, the regression curves obtained with endothall and cantharidin (Fig. 4B) support the time-dependent inactivation of serine/threonine PP. However, the lines were not completely parallel in comparison to the control, which may be due to the fact that assays were performed in crude extracts. Indeed plants have a multitude of serine/threonine PP, and some of these PPs by endothall or cantharidin. As no equilibrium can be established and inhibition of serine/threonine PP activity increases over time, the dose–response curves can only yield I$_{50}$ app, because this value will be time-dependent. Nonetheless, the dose–response curves were performed carefully to ensure that the incubation time with the inhibitor and the subsequent enzymatic assays lasted precisely the same duration for each experiment. Under these conditions, the I$_{50}$ app values obtained were highly reproducible and were used as a relative value to compare the potency of endothall and cantharidin.

The inhibition of serine/threonine PPs by cantharidin at an IC$_{30}$ dose based on chlorophyll content caused significant changes in about 10% of the *A. thaliana* transcriptome [31]. Serine/threonine PPs control many signaling processes associated with hormones, light, and response to pathogens and stress. Proper function of these processes is essential for plant survival. Thus, inhibition of several of these critical PPs by endothall causes signaling disruption that can be lethal at high enough doses of the herbicide.

While the potency of endothall on serine/threonine PP activity was lower (Fig. 3) and its binding appeared to develop more slowly (Fig. 4) than that observed with cantharidin, it had a more pronounced effect on the expression of genes than cantharidin (Fig. 5). The general effects of these two serine/threonine PP inhibitors on transcription of the eleven genes selected for examination were similar. Both compounds caused an initial change in expression, which resulted in a high Pearson correlation coefficient (Fig. 5A). However, the effect of cantharidin began to subside after 10 h, whereas the effect of endothall continued to be accentuated (Fig. 5B). This suggests that the commercial herbicide remains bioactive for a longer period of time than the natural product in *planta*. The effect of endothall on transcription of these genes was still higher than cantharidin after 24 h exposure. Nevertheless, the phytotoxicity of cantharidin to *A. thaliana* when sprayed onto plants was slightly higher than endothall (Fig. 2A). Examination of another set of the many genes affected by cantharidin [31,32] may have given different relative results, but we expect that the general effects of the two compounds would be similar.

The complete inhibition of serine/threonine PP at the highest concentrations of both inhibitors (Fig. 3) indicates that all serine/threonine PPs in the plants are inhibited. This and the similar relative phytotoxicity and enzyme inhibition (Figs. 2 and 3) provide strong evidence that the herbicide mode of action of endothall is intimately related to inhibition of serine/threonine PP. Lastly, since there are at least 19 serine/threonine PPs in *A. thaliana*, one would expect multiple forms of the endothall susceptible enzyme to be found in all plant species. Thus, evolution of resistance to endothall at the target site is highly unlikely.

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**References**


